Effect of *Epichloë* endophyte strains in *Lolium* spp. cultivars on Argentine stem weevil parasitism by *Microctonus hyperodae.*

S.L. Goldson[1,2], F. Tomasetto[1] and A.J. Popay[3]

[1]AgResearch Ltd, Private Bag 4749, Christchurch 8140, New Zealand
[2]Bio-Protection Research Centre, PO Box 85084, Lincoln University, Lincoln 7647, New Zealand
[3]AgResearch Ruakura, Private Bag 3123, Hamilton 3240, New Zealand
Corresponding author stephen.goldson@agresearch.co.nz

Abstract This paper reports on an extensive field investigation conducted at Lincoln during the 2014-2015 summer/early autumn. This aimed to assess the effects of a range of novel *Epichloë* endophytes when present in different cultivars of *Lolium* spp. on parasitism rates by the biological control agent, *Microctonus hyperodae*, in *Listronotus bonariensis* (i.e. the Argentine stem weevil). Results for the entire summer, and including all treatment combinations, did not find any significant differences in parasitism in *L. bonariensis* populations. However, in the early autumn, independent of the endophytes present, significantly higher levels of parasitism were found in a tetraploid *Lolium multiflorum* cultivar and a tetraploid *L. perenne* selection compared to the *L. perenne* cultivars. Whether this finding has any bearing on a possible mechanism of weevil resistance is discussed.

Keywords Argentine stem weevil, decline, *Lolium multiflorum*, *Lolium perenne*, parasitism rates, parasitoid, pasture pest, resistance.

INTRODUCTION

Over recent years concern about the impact of the Argentine stem weevil (*Listronotus bonariensis*) on *Lolium*-based pasture has been significantly reduced by using selected strains of *Epichloë* endophytes in ryegrass pastures (Johnson et al. 2013) and by the significant impact of the braconid parasitoid biological control agent *Microctonus hyperodae* (e.g. Barker & Addison 2006). At the same time, attention has been diverted towards the severe damage occurring in white clover by the clover root weevil, *Sitona obsoletus* (e.g. Gerard et al. 2009; Ferguson et al. 2012).

However, recent evidence indicates that *M. hyperodae* has been losing its efficacy. Widespread and diverse sources of New Zealand data on *L. bonariensis* parasitism have permitted meta-analyses that have shown that levels have dropped by more than 50% in *Lolium*-based pastures in the last 10-15 years (e.g. Goldson et al. 2014a, 2014b). Further, this has coincided with reports of increased pasture damage by the weevil (e.g. Popay et al. 2011).

It is tempting to attribute this downward parasitism trend to resistance developing in the weevil to the parasitoid from continuous and high selection pressure over the last 20 or so years. This is particularly given the parthenogenetic reproduction by the parasitoid and the sexual reproduction by the weevil resulting in what is sometimes termed an 'unequal evolutionary arms-race'. While the decline is very apparent, the underlying mechanism is unknown. As well as the obvious suggestion of genetic change through selection pressure, there are possibly other mechanisms including changed farming practice and the influence of nationwide adoption of novel animal-friendly endophytes in *L. perenne*.

Earlier studies have shown mixed results on the effect of endophytes on *M. hyperodae* development and survival. In a laboratory study, Barker & Addison (1996) showed that *M. hyperodae* larval development was retarded in *L. bonariensis* fed on endophyte-infected *L. perenne*. Furthermore, Goldson et al. (2000), in a short-term field study using *L. perenne* infected with either AR6 or common-toxic (CT) endophytes, showed that there was a significant inverse relationship between parasitism levels and the alkaloid peramine. Additionally, Bultman et al. (2003) conducted *L. bonariensis* laboratory feeding trials on *L. perenne* infected with CT, AR1, AR6 and AR37 endophytes. Here they showed that CT endophytes and AR6 significantly reduced adult parasitoid emergence compared to the control. Conversely, AR37 had no effect on parasitoid survival and indeed appeared to accelerate parasitoid development compared to the other strains (Bultman et al. 2003).

While the work reviewed above showed, in laboratory and short term field trials, mixed effect of *Epichloë* endophytes on *M. hyperodae* development and survival, the primary question remained as to whether industry adoption of new *Lolium* endophyte strains could have influenced the observed major declines in parasitism (Goldson et al. 2014a, 2014b). In order to assess this, a large study was conducted throughout the 2014-2015 summer/early autumn season; the results of this are presented in this paper.

METHODS AND MATERIALS

This study was based on a large programme on the AgResearch Lincoln Research Farm originally designed to test the effects of endophytes on sheep staggers (AgResearch endophyte trial site; -43.631788, 172.464938). Another smaller seed-production area on the Lincoln Research Farm was also used (Grasslands Innovation nucleus seed production site; -43.628221, 172.453382).

AgResearch endophyte trial site

Twenty trial plots, 35 × 50 m, were sown on September 2013 at the AgResearch Research Farm. The *L. perenne* plots were sown at 20 kg/ha and the *L. multiflorum* plots at 25 kg/ha; all established well. These plots were set-stocked with lambs from late January until mid-April 2014. Nitrogen was applied as urea at 50 kg/ha on one occasion in spring 2013 and on two occasions in summer/autumn 2014. Throughout the winters, the plots were rotationally grazed with hoggets. Cropmaster 15 (N, P, K & S (Ravensdown)) was applied in early October 2014 at 300 kg/ha and in early December 2014 nitrogen was applied as urea (80 kg/ha); after this, the pasture was conserved and removed. In early January 2015 urea was applied again at 100 kg/ha. In mid-January the plots were topped to remove seed heads and normalise pasture cover for grazing.

Endophyte infection percentages in the plots as tested in the 2013/2014 season were >85% for all treatments. All plots were periodically sprayed with Puma S (fenoxaprop-P-ethyl, Bayer), which resulted in all plots comprising 100% ryegrass by late January 2015. Weather conditions over summer 2014-2015 led to exceptionally high levels of alkaloids, resulting in a high level of ryegrass staggers on the CT endophyte treatment. The trial was irrigated as part of the normal farm rotation.

Grasslands Innovation nucleus seed production site

The Grasslands Innovation seed crop comprised two 30 × 30 m, juxtapositioned plots of *L. multiflorum* (cv. Lush). These were drilled in mid-March 2014; one plot was inoculated with AR37 while the other was endophyte free. The former was drilled at 13 kg/ha and the latter 6.5 kg/ha to compensate for different germination rates. The resulting pure swards looked identical. The plots were maintained to maximise seed production with regular urea application and topping to prevent too much vegetative production. The plots were mulched in mid-September 2014 and the seed harvested in late January 2015.

Sampling regime

The experiment consisted of a three replicate randomised block design comprising the following eight treatments: (1) cv. Samson (diploid perennial) endophyte free, (2) cv. Samson (diploid perennial) CT, (3) cv. Samson (diploid perennial) AR1, (4) cv. Samson (diploid perennial) AR37, (5) cv. Rely (diploid perennial) AR37, (6) unnamed selection (Code KLp902) (tetraploid perennial) AR5, (7) cv. Lush (tetraploid Italian) AR37 and (8) cv. Lush (tetraploid Italian) endophyte free.

All plots were randomly sampled fortnightly at the Grasslands Innovation site and monthly at the AgResearch Research Farm site from 8 October 2014 until 7 April 2015 using a modified leaf-blower vacuum (Echo 21cc) to suck the insects into a removable net recessed in the inlet pipe. Collections were made by dragging the machine across each plot for 15 min. The weevils were then removed from the litter and dissected to determine levels of parasitism. A minimum sample size of weevils for dissection was set at >14 occasionally leaving some gaps in the data. On such occasions resampling of plots with very low weevil densities was not possible given the scale of the study, but the repeated sampling of the plots and the number of replicates minimised any effect.

Statistical analysis

To test the significance of any differences between the mean parasitism levels in the endophyte strains and cultivars of *L. perenne* and *L. multiflorum* and differences in parasitism rates between the *L. perenne* diploid and *L. perenne* tetraploid treatments, analyses of variance (ANOVA) and post-hoc Tukey's HSD tests were conducted using R 3.2.0 (R Development Core Team, 2015).

Moreover, the multiple time-series data obtained were analysed using a cross-correlation analysis built in the 'tseries' package (Trapletti & Hornik 2015). This analysis measured the extent of similarity of two series (Cross-correlation = 1) as a function of the lag of one relative to the other and the analysis was based on a normalised cross-covariance function (Cowpertwait & Metcalfe 2009). This allowed estimation of the levels of curve similarity.

RESULTS

Figure 1 shows the prevailing levels of parasitism at monthly intervals during the 2014-2015 summer-early autumn months. Notably, the monthly sampling does not accommodate the wild fluctuations in levels of parasitism that occur at this time due to the interacting population dynamic processes of both the weevils and the parasitoid (e.g. Goldson et al. 2014a). Irrespective, there were no significant differences in the levels of parasitism across the various grass types (Cross-correlation = 0.89, $P<0.001$). Similarly, Figure 2 at fortnightly intervals shows no significant effect of AR37 in cv. Lush (Cross-correlation = 0.85, $P<0.001$). Considering the *L. perenne* and *L. multiflorum* cultivar parasitism patterns across the entire season, there was no significant difference in the trends throughout the summer sampling period (Cross-correlation = 0.75, $P<0.05$). However, this changed abruptly around the time of the last sampling event (7 April 2015) when significantly higher mean parasitism levels occurred in the *L. multiflorum* plots (55.8% ± 0.8%) than the *L. perenne* plots (43.3% ± 1.7%, $P<0.001$; Table 1). On the same date the tetraploid *L. perenne* (KLp902) plots showed higher parasitism (48.5% ± 1.5%) versus their non-tetraploid counterparts (41.5% ± 1.6%, n.s.; Table 2).

DISCUSSION

Endophyte effects

The lack of any endophyte treatment effects on parasitism across the whole season was in spite of the summer's unusually dry conditions accentuating endophyte alkaloid expression such that severe ryegrass staggers occurred in the CT endophyte treatment (L. Sutherland, AgResearch, personal communication). Notably, these results are at variance with the earlier observations of Barker & Addison (1997), Goldson et al. (2000)

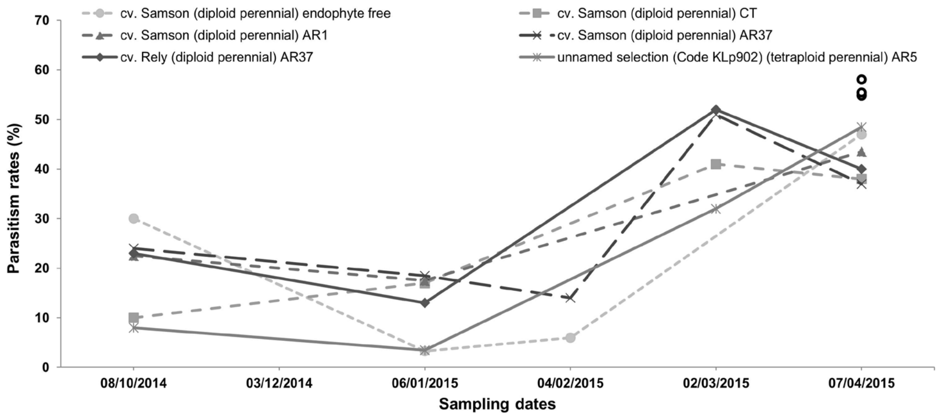

Figure 1 *Microctonus hyperodae* parasitism (%) as measured in *Listronotus bonariensis* collected between 8 October 2014 and 7 April 2015 from plots containing different ryegrass species and endophytes at the AgResearch Research Farm at Lincoln. Significantly higher levels of parasitism in tetraploid *Lolium multiflorum* plots are shown as single points (⭘) at the 7 April 2015 sampling. Values are the mean of >14 samples. Across the entire experiment there were no significant treatment differences in *M. hyperodae* parasitism levels within the *Lolium perenne* plots.

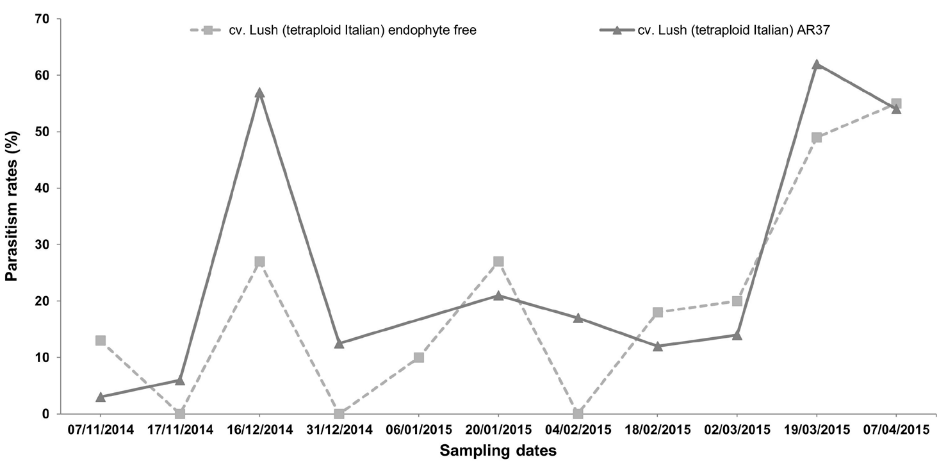

Figure 2 *Microctonus hyperodae* parasitism (%) as measured in *Listronotus bonariensis* collected between 8 October 2014 and 7 April 2015 from plots containing tetraploid *Lolium multiflorum* and endophyte at the Grasslands Innovation nucleus seed production site at Lincoln. Values are the mean of >14 samples. Across the entire experiment there were no significant treatment differences in *M. hyperodae* parasitism levels within the *L. multiflorum* plots.

Table 1 *Microctonus hyperodae* parasitism rates (%) as measured in >14 samples. *Listronotus bonariensis* collected from all of the *Lolium perenne* and tetraploid *Lolium multiflorum* plots on 7 April 2015. The mean parasitism rate was significantly higher in the tetraploid *L. multiflorum* plots (*** P<0.001).

L. perenne		*L. multiflorum*	
cv. Rely AR37	40	cv. Lush endophyte-free[1]	55
cv. Samson endophyte-free	47	cv. Lush AR37[1]	54
cv. Samson CT	38	cv. Lush AR37 (Rep 1)	57
cv. Samson AR1 (Rep 1)	44	cv. Lush AR37 (Rep 2)	57
cv. Samson AR1 (Rep 2)	43		
cv. Samson AR37	37		
cv. KLp902 AR5 (Rep 1)	47		
cv. KLp902 AR5 (Rep 2)	50		
Mean	**43.3**		**55.8*****

[1]The Grasslands Innovation nucleus seed production site

Table 2 *Microctonus hyperodae* parasitism rates (%) as measured in >14 samples. *Listronotus bonariensis* collected from all the diploid *Lolium perenne* and tetraploid *L. perenne* plots on 7 April 2015. The mean parasitism rate was not significantly different.

Diploid		Tetraploid	
cv. Rely AR37	40	cv. KLp902 AR5 (Rep 1)	47
cv. Samson endophyte-free	47	cv. KLp902 AR5 (Rep 2)	50
cv. Samson CT	38		
cv. Samson AR1 (Rep 1)	44		
cv. Samson AR1 (Rep 2)	43		
cv. Samson AR37	37		
Mean	**41.5**		**48.5**

and Bultman et al. (2003). However, the results obtained by these authors were often based on laboratory studies (Barker & Addison 1997; Bultman et al. 2003) and the work by Goldson et al. (2000) involved only two summer sampling dates. In general therefore, at the very least, the results of this study strongly indicate that the observed nationwide decline in parasitism over the last ca 20 years (Goldson et al. 2014a, 2014b) has not been caused by the widespread adoption of new endophyte strains. That the pattern of *M. hyperodae* parasitism rates in the *L. multiflorum* plots across the entire season was not significantly different from that found in the *L. perenne* plots was somewhat contrary to expectation.

Early autumnal parasitoid parasitism rates

The early autumnal appearance of cultivar-related differential parasitoid parasitism rates could have been due to the occurrence of significant plant growth and the recovery of weevil populations from generally low numbers. The hierarchy of parasitism levels, with the highest in the tetraploid *L. multiflorum*, then the tetraploid *L. perenne* plots and lowest in the *L. perenne* cultivars, is similar to patterns found elsewhere. McNeill et al.'s (2007) Wairarapa biocontrol study showed that the *M. hyperodae* level in cv. Quartet AR1 (tetraploid *L. perenne*) pasture was 51% compared to a mean of 34% in cv. Aires (diploid *L. perenne*). Similarly, recent

Waikato autumnal data again have shown the same pattern with higher levels of *M. hyperodae* parasitism in *L. multiflorum* pastures than their *L. perenne* counterparts (P.J. Gerard, AgResearch, personal communication). Further work is planned at Lincoln to investigate these trends during the 2015 winter.

Implications of varied autumnal parasitism rates

Several hypotheses can be proposed to explain why *M. hyperodae* parasitism rates were higher in the tetraploid *L. multiflorum* plots than the diploid *L. perenne* plots. Certainly the weevil prefers tetraploid *L. multiflorum* plants to diploid *L. perenne* plants (e.g. Goldson 1982; Barker 1989) and therefore the resulting higher levels of feeding may lead to higher levels of parasitoid attack (Phillips 2002). However, in this study the results may also provide clues as to the nature of the New Zealand-wide parasitism decline, especially with regard to the possibility of acquired resistance to parasitoid attack by *L. bonariensis*. In the absence of any observed signs of physiological resistance to the parasitoid (e.g. encapsulation; S.L. Goldson, unpublished data), a parsimonious explanation for the reduced parasitism could be the selection for a behavioural shift in the weevil such that parasitoid evasion has become enhanced. Such adaptation by *L. bonariensis* could be founded on earlier observations that have shown that effective parasitoid attacks tend to occur most often during weevil feeding and grooming; at these times the species' mouth and caudal end become accessible for parasitoid oviposition (Phillips 2002). Also, *L. bonariensis* populations tend to be less prevalent on the foliage in the presence of *M. hyperodae* (Gerard 2000). Arguably, if selection pressure has led to an enhancement of parasitoid-avoiding behaviours based on these characteristics, it would have developed mostly in the context of the country's extensive diploid *L. perenne* pastures and would therefore be less likely to be expressed in the relatively rare tetraploid plantings. Such an effect may well be based on nothing more than the differences in the plant architecture between the tetraploid and diploid ecosystems. This is supported by Phillips' (2002) observation where the orientation of plant material in a cage could influence parasitoid efficacy. Furthermore, differences in plant architecture are likely to be most apparent during autumnal regrowth when diverging parasitism levels were observed in this study.

There could also be a nutritionally-based explanation for the differences whereby the tetraploids have superior nutritive qualities (Sun et al. 2010) that could enhance parasitoid fitness and parasitism rates. Contrary to this though, the endophytes used in this experiment with typical *L. bonariensis* antifeedant qualities had no significant effect on parasitism rates. Thus nutrition is unlikely to be the mechanism behind the observed declines in *L. bonariensis* parasitism.

Confounding effects of *M. aethiopoides* parasitism on *L. bonariensis*

Finally, given the nature of this study, it is possible that opportunist parasitism of *L. bonariensis* by the *Sitona* spp. parasitoid (*M. aethiopoides*; e.g. McNeill et al. 2002), could have affected some of the results, particularly at times when the *M. aethiopoides*:host ratios were biased towards the parasitoid. This can typically occur in autumn (S. Hardwick, AgResearch, unpublished data). Thus, it may be that at certain times during this study the levels of *L. bonariensis* parasitised by *M. hyperodae* could have been over-estimated. Nonetheless, the effect of *M. aethiopoides* in the *L. multiflorum* plots at the Grasslands Innovation nucleus seed production site is likely to have been minimal as the sward comprised only *L. multiflorum,* with no white clover whatsoever to support the population dynamics discussed above.

CONCLUSION

This study has shown conclusively that, given the non-significant effects of novel *Epichloë* endophytes on parasitism of *L. bonariensis* by *M. hyperodae,* the endophytes have not been the reason for a decline in the biological control of *L. bonariensis*. The apparently varied impacts of *Lolium* spp. on early autumnal parasitism may be starting to provide some clues to the mechanisms behind parasitism decline.

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